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CELL CYCLE KINETICS AND DEVELOPMENT OF *HYDRA ATTENUATA*

I. EPITHELIAL CELLS

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SUMMARY

The cell cycle parameters of epithelial cells of *Hydra attenuata* are described. Specifically the rate of proliferation and the fraction of proliferating cells have been determined under conditions of defined growth rate. Techniques involved standard methods of cell cycle analysis using histological and tissue maceration preparations; pulse-chase and continuous labelling with [^{3}H]thymidine followed by autoradiographic analysis, and microspectrophotometric determination of nuclear DNA content in single cells. The results indicate that more than 90% of hydra epithelial cells are actively proliferating with a cell cycle duration about equal to the tissue doubling time. In well fed hydra the average cell cycle is about 3 days long. *S* period is 12–15 h, G_1 0–1 h, and mitosis 1·5 h. Most of the cell cycle consists of a long G_2 period of variable duration (24–72 h). The results provide no evidence for a subpopulation of rapidly proliferating cells as predicted by 'growth zone' models of hydra morphogenesis. The results also indicate that the population of epithelial cells is self-sustaining requiring no input by differentiation from other cell types. The long and variable G_2 period means that DNA synthesis and the following cell division are effectively uncoupled such that inhibitors of DNA synthesis may not stop epithelial cell division. The variable nature of the G_2 period suggests it as a possible point of control of hydra growth.

INTRODUCTION

The small freshwater coelenterate *Hydra*, because of its morphological simplicity and limited diversity of cell types, is useful for studying the control mechanisms underlying the organization of cells into a defined multicellular complex. For such a study, basic information about the kinetics of proliferation and differentiation for the various cell types is required. In the present paper the epithelial cells – the principal structural element of hydra tissue – are characterized with respect to their proliferation rate and cell cycle parameters.

Hydra tissue consists of 2 concentric cylinders of epithelial cells (endoderm and ectoderm) enclosing a central gastric cavity. A number of other cell types (nerves, interstitial cells, nematoblasts, gland cells) are scattered, clustered or singly, between these epithelial cells. The tissue grows continuously, while maintaining a constant size and cellular composition. This steady-state condition is achieved principally by

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the shedding of tissue in the form of buds from the proximal body region and to a lesser extent by the sloughing of tissue at the distal and proximal ends of the animal.

Two hypotheses have been put forward to explain the relationship between growth and morphogenesis in hydra. Brien & Reniers-Decoen (1949) and Burnett (1961) postulated the existence of a localized region of cell proliferation proximal to the hypostome. This subhypostomal growth zone was considered to determine hydra's form. Campbell (1967*a*) and Clarkson & Wolpert (1967) have proposed that epithelial cell proliferation occurs generally throughout hydra tissue and that the animal's characteristic morphology is caused by mechanisms other than growth (e.g. cell and tissue movement.) The first hypothesis predicts that a small fraction of epithelial cells proliferates more rapidly than the animal grows, while the second predicts that the whole epithelial cell population proliferates at a rate comparable to the hydra's growth rate. The extent and possible function of epithelial cell production by differentiation from a precursor cell, as suggested by morphological evidence (Brien & Reniers-Decoen, 1949; Lentz, 1966), is unknown although it could be quite significant in such morphogenetic events as budding and regeneration.

In the present paper the proliferation of hydra epithelial cells has been studied by standard methods of cell cycle analysis (e.g. Cleaver, 1967) using preparations of single cells obtained by quantitative maceration of hydra tissue (David, 1972). The results indicate that most epithelial cells proliferate at a rate comparable to the growth rate of hydra. The results are not consistent with the hypothesis of a subhypostomal growth zone nor do they indicate a significant production of epithelial cells by differentiation. The results demonstrate further that hydra epithelial cells have an unusually long and variable premitotic (G_2) period which is important to recognize when studying hydra development experimentally.

MATERIALS AND METHODS

Hydra attenuata (Pallas) were used in all experiments. The original strain was obtained from Professor P. Tardent, Zürich, in 1966, and has been cultured asexually in our laboratory since then. The hydra used were large 'steady-state' animals (Campbell, 1967*a*) obtained by culture at low population density (1–2 hydra per ml medium) with heavy feeding. Hydra were fed excess numbers of freshly hatched *Artemia* nauplii once daily at 10 a.m., gently transferred to a clean dish after 1 h, and washed 6 h later. Cultures were maintained in closed plastic Petri dishes (25 cm diameter) in a temperature-controlled incubator (20–21 °C). Under these conditions hydra have a population doubling time of 3·2–3·5 days (Fig. 1, p. 560). Population doubling time was measured in a culture with all animal sizes and ages present in normal proportions; thus a doubling in hydra number probably reflects a doubling of tissue mass.

Large steady-state hydra contain about 24000 epithelial cells (not including buds); of these, 22000 are in the body column and 2000 in the tentacles. In all experiments tentacles and buds were removed before assaying the hydra. In some experiments the body column was subdivided into 3 sections: hypostomal region, gastric region, and peduncle-basal disk, containing about 2500, 16500, and 3000 epithelial cells, respectively (Bode *et al.* 1972).

Hydra were labelled with [*Me*-^{3}H]thymidine (20 Ci/mM), or [2-^{14}C]thymidine (50 μCi/mM) (The Radiochemical Centre, Amersham, England). Both compounds were used at concentrations of 2–50 μCi/ml depending on the experiment. The radioactive thymidine was administered by injecting about 0·2 μl into the gastric cavity of individual hydra, using a 10-μl Hamilton syringe fitted with a tip of finely drawn polyethylene tubing. A single injection resulted in a pulse label of 15–30 min duration in unfed hydra (C. N. David, unpublished). 'Continuous'

labelling was achieved by repeated injections of radioactive thymidine at intervals shorter than the length of the *S* period of epithelial cells, usually at about 8-h intervals.

The maceration technique for quantitatively preparing single cell suspensions from hydra tissue and the identification of epithelial cells in such suspensions have been described (David, 1972). For autoradiography, cell suspensions were fixed with 4% formalin and 0·02% osmium tetroxide in maceration fluid before spreading on gelatin-coated microscope slides. After drying overnight the slides were washed briefly to remove excess maceration fluid and fixative, and then were covered with Kodak AR-10 stripping film. Autoradiographs were exposed for 1–2 weeks (4 weeks when a single 2 μCi/ml isotope application was made). Double-label autoradiography for the detection of ^{3}H and ^{14}C in the same cells was performed by a 2-emulsion layer technique (Burns, 1972). Slides were dipped in Ilford K-5 nuclear emulsion diluted 1:1 with water, exposed for 1 week and developed. The slides were then coated with a film of 7·5% nitrocellulose and finally dipped in undiluted Ilford emulsion. The second film layer was exposed for 2–3 weeks and developed. Cells containing only ^{3}H exposed only the lower film layer; cells containing ^{14}C exposed primarily the upper film layer.

Autoradiographs were analysed using bright-field and phase-contrast microscopy. The labelling index (labelled epithelial nuclei/total epithelial nuclei) was determined by counting 200–500 epithelial nuclei per sample. To minimize errors due to variability between animals, 3–5 hydra were used for every sample except where stated otherwise.

For assessing the degree to which mitotic cells were labelled, maceration preparations and 8-μm histological cross-sections were used. Both were stained by the Feulgen method (Romeis, 1948) and the positions of mitotic cells were recorded. The mapped mitotic figures were subsequently analysed for labelling by autoradiography. This procedure was used in order to avoid bias as to degree of isotope labelling when locating mitotic cells.

Determination of the amount of DNA per nucleus for identification of G_1 ($2n$) and G_2 ($4n$) cells was performed using Feulgen staining and quantitative absorption measurements (Zeiss scanning microspectrophotometer UMSP 1) or using fluorescent Feulgen staining with 2,5-bis-[4′-amino-phenyl-(1′)]-1,3,4 oxdiazole and quantitative fluorescence measurements (Ruch, 1966). The 2 methods gave similar results. In some cases, cell suspensions obtained by maceration were vigorously pipetted to break the epithelial cells and free their nuclei before preparing slides. This technique improved the accuracy of the measurements by eliminating cytoplasmic background. Using bovine lymphocytes as a reference (7×10^{-12} g DNA per nucleus; P. Hausen, personal communication), the diploid nuclear DNA content of *Hydra attenuata* was determined to be $3{\cdot}5 \times 10^{-12}$ g. This value is in agreement with the results of chemical determinations of $3–3{\cdot}4 \times 10^{-12}$ g DNA per nucleus (H. Bode, unpublished).

For mitotic counts histological sections were made on sets of 2 hydra fixed in Lavdowsky's fixative (acetic acid:formalin:ethanol:water 5:10:30:55), embedded in paraffin, sectioned at 8 μm, stained by the Feulgen method with light green counter stain and mounted in Permount.

RESULTS

Proliferative fraction of the epithelial cell population

To determine the fraction of proliferating epithelial cells, hydra were continuously labelled with [^{3}H]thymidine for 5 days. Several animals were assayed each day by maceration and autoradiography to determine the labelling index in the epithelial cells. The results for the hypostomal region, gastric region, and peduncle-basal disk are given in Fig. 2. Most epithelial cells in the gastric region and hypostomal region of hydra pass through *S* period at least once in 3 days. About 70% of the peduncle-basal disk epithelial cells similarly pass through the *S* period in 3 days. The remaining peduncle-basal disk epithelial cells remain unlabelled for at least 5 days. Taking into account the absolute numbers of epithelial cells in each region (hypostome 2500, gastric column 16500, peduncle 3000 (Bode *et al.* 1972)) these results indicate that more than 90% of all epithelial cells in the body column of hydra are proliferating.

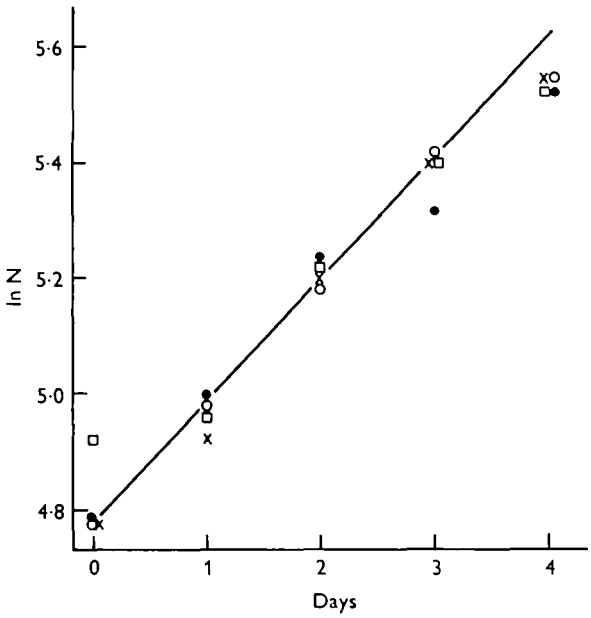

Fig. 1. Growth rate of *Hydra attenuata*. 25-cm plastic Petri dishes were inoculated at a population density of 1–2 hydra/ml with a random selection of individuals of all sizes from a rapidly growing culture. Thereafter, the hydra were heavily fed once daily and maintained at 20–21 °C. The number of individuals was counted daily (a hydra with several buds was counted as one individual; each bud after it had fallen off was counted as one individual). Ordinate: logarithm of the number of individuals. Abscissa: time in days. Data from 4 independent experiments are shown. Curve indicates a doubling time of 3·3 days.

The cell cycle is about 3 days for these cells which is similar to the population doubling time (3·3 days, Fig. 1).

DNA content of epithelial cell nuclei

Based on the above results, further information about the relative durations of the different periods of the cell cycle can be obtained by determining the relative numbers of epithelial cells with $2n$ (G_1 period), $2n$–$4n$ (S period) or $4n$ (G_2 period) DNA contents. Fig. 3 shows the distribution of nuclear DNA contents of 100 epithelial cells. The majority have $4n$ DNA contents and are thus in the G_2 period of the cell cycle. A small fraction of the cells are in the S period ($2n$–$4n$) and essentially no cells are found in the G_1 period. From the ratio of G_2 cells to total cells one estimates that the duration of the G_2 period of the epithelial cell cycle is 5–7 times longer than the G_1 and S periods combined.

The identification of S and G_2 cells was confirmed by measuring DNA content and [^{3}H]thymidine incorporation (by autoradiography) on the same nuclei in hydra pulsed for 30 min with [^{3}H]thymidine before maceration. Of 9 nuclei having DNA contents between $2n$ and $4n$, 7 were labelled; of 13 nuclei with $4n$ DNA content, only one was labelled.

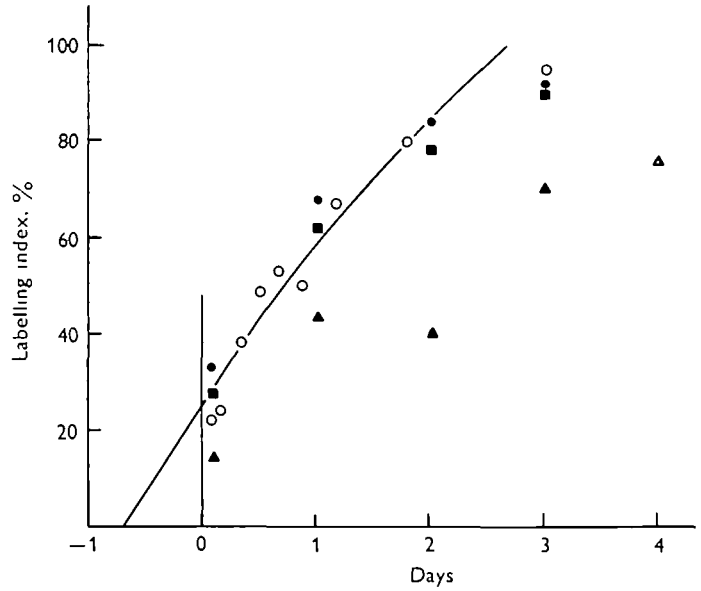

Fig. 2. Labelling index of epithelial cells in hydra continuously labelled with [^{3}H]-thymidine. 50 hydra were injected daily at 8 a.m., 4 p.m. and 12 midnight with [^{3}H]thymidine (2 or 50 μCi/ml) during the experiment and otherwise cultured as usual. 3–5 hydra were sampled at each time point; hydra to be sampled were labelled with [^{3}H]thymidine for 30 min immediately before preparing macerations. Data are presented from 2 independent experiments. Symbols represent different parts of body column assayed separately. ○, Entire body column minus hypostome and peduncle-basal disk; ●, lower half of gastric column including budding region; ■, hypostome and upper half of gastric column; ▲, peduncle-basal disk. The curve is the theoretical result for an exponentially growing population of cells having a cell cycle of 3·3 days (G_1 = 0, S = 16, G_2 = 64 h).

Diurnal fluctuation in mitotic and labelling indices

Heavy feeding induces partial synchrony in the epithelial cell population of hydra (Campbell, 1967*a*). Fig. 4 shows the variation in mitotic index and labelling index over a 24-h period in steady-state hydra fed daily at 10 a.m. There is a sharp increase in epithelial cell mitotic figures 12 h after feeding followed immediately by an increased labelling index.

G_1 period

In a synchronized cell population the duration of the G_1 period can be estimated from the time difference between a peak in the mitotic index and the subsequent rise in the labelling index. The data in Fig. 4 indicate this time difference for epithelial cells to be less than 2 h. Since this time difference also consists partly of mitosis, it is possible that these cells have no G_1 period.

The short duration of G_1 is in agreement with the low frequency of epithelial cells having a $2n$ DNA content. Furthermore, the presence of a peak in the labelling index following the mitotic index peak indicates that most or all epithelial cells must have

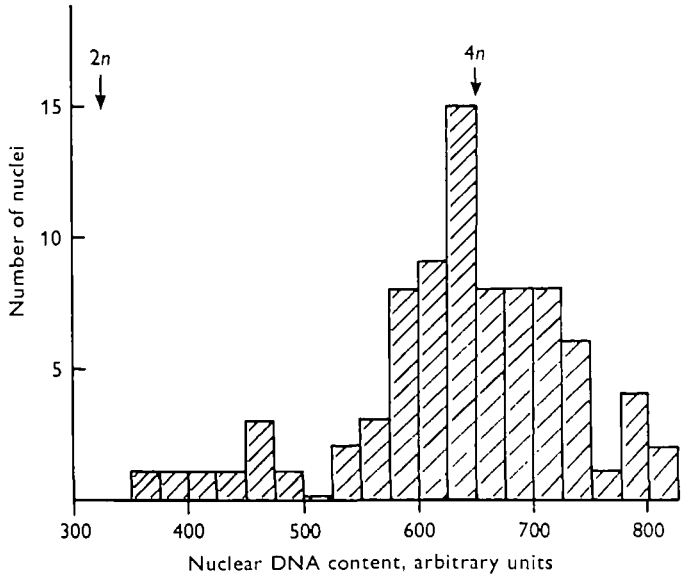

Fig. 3. Nuclear DNA contents of epithelial cells, as determined by quantitative fluorescence measurements on single nuclei stained with the fluorescent Feulgen reagent bisaminophenyl-oxdiazole. Cells were prepared by macerating hydra at 2 p.m. Standard $2n$ and $4n$ DNA values were determined from mitotic figures (telophase and metaphase). Ordinate: number of nuclei. Abscissa: nuclear DNA content in relative units of fluorescence.

the short G_1 period. Any dispersion in the duration of G_1 would significantly reduce and broaden the peak in the labelling index.

S period

The duration of the S period was determined by the use of the [^{3}H]thymidine-[^{14}C]thymidine double-labelling technique (Wimber & Quastler, 1963). To avoid errors introduced by a non-uniform distribution of cells within the S period (due to partial synchrony of the epithelial cell population) the cited procedure was modified. An initial pulse of [^{3}H]thymidine was given, followed by a second pulse of [^{14}C]-thymidine after varying intervals of time. Cells which had passed from S to G_2 in the interval between the ^{3}H- and ^{14}C-pulses were labelled only with ^{3}H; all other ^{3}H-labelled cells were 'overlabelled' with ^{14}C by the second pulse. The time required for all ^{3}H-labelled cells to become free of ^{14}C-overlabelling corresponds to the duration of the S period.

The results of such an experiment are given in Fig. 5. As the interval between the ^{3}H- and ^{14}C-pulses increased, the percentage of cells labelled only with ^{3}H increased until a maximum was reached. The time to attain this maximum indicates that the duration of the S period is 12–15 h.

At the time of the ^{3}H-pulse (9 a.m.), more cells in late S period were labelled than cells in early S period, due to the partial synchrony in the epithelial cell population. As a result the curve for ^{3}H-labelled cells in Fig. 5 showed a rapid increase at first and then slowly attained a maximum. This non-linear increase does not, however, affect

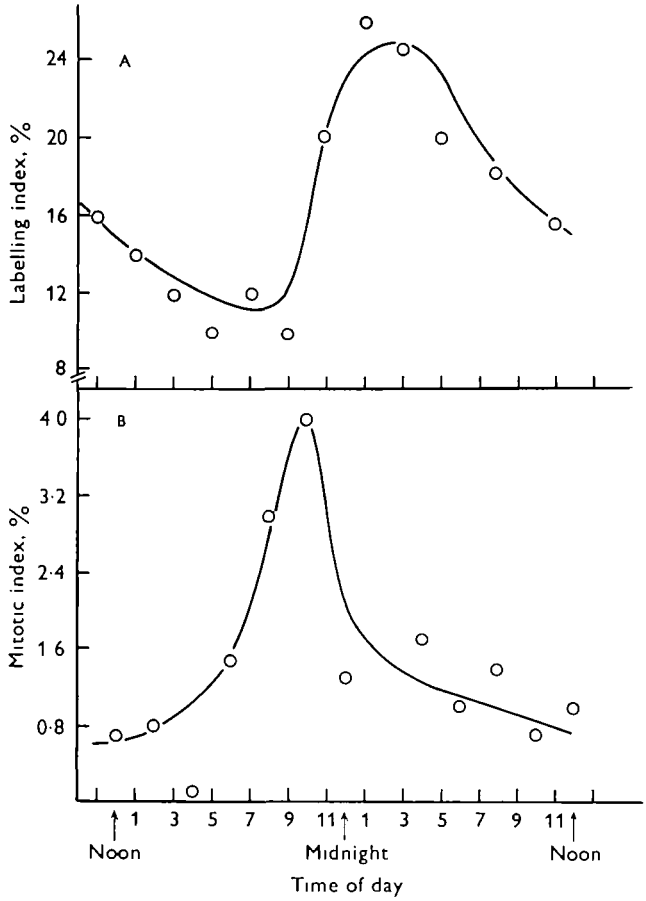

Fig. 4. Temporal variation in labelling index (A) and mitotic index (B). Labelling index of epithelial cells was determined in macerated preparations of labelled hydra by autoradiography. At each of the times shown, 2 hydra were pulse-labelled for 30 min with [^{3}H]thymidine (200 μCi/ml) and immediately sampled by maceration. The mitotic index was determined in histological sections of specimens taken simultaneously from the same culture. Hydra were fed at 10 a.m. before the start of the experiment. Ordinate: mitotic index (%) and labelling index (%). Abscissa: time of day.

the determination of the S period duration which is based on the time to attain a maximum. The results are also not perturbed by ^{3}H-labelled cells undergoing mitosis because the minimum G_2 period for epithelial cells is 24 h (see below).

It has been possible to estimate independently the duration of the S period from the continuous labelling data in Fig. 2. For the special case of cells with a G_1 period equal to 0 h, the labelling index curve intersects the abscissa at the negative value of the duration of S period (C. N. David, unpublished). In this way from Fig. 2 one estimates the duration of the S period as 12–16 h. A more precise determination was not possible due to errors in the determination of labelling index and irregularities introduced by the partial synchrony of epithelial cells. However, the estimate of 12–16 h agrees well with the value determined from the double-labelling experiment.

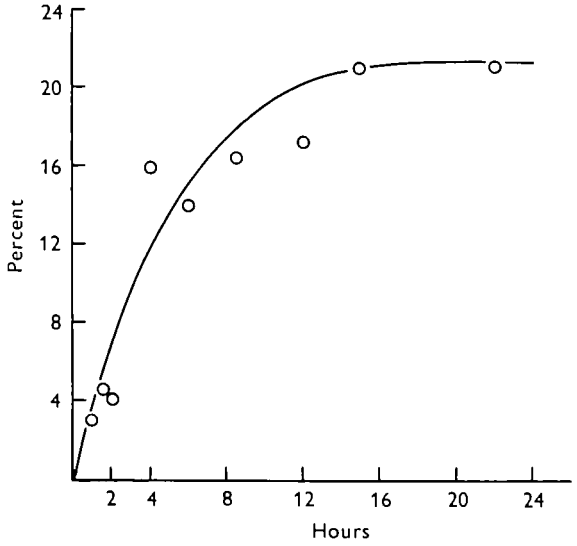

Fig. 5. Determination of S period duration by [^{3}H]- and [^{14}C]thymidine double-labelling technique. 30 hydra were injected with [^{3}H]thymidine (50 μCi/ml) at 9 a.m. At varying intervals thereafter, 3 hydra were injected with [^{14}C]thymidine (50 μCi/ml) and macerated 60 min later. Two-emulsion autoradiography was performed to distinguish between ^{3}H-labelled and ^{14}C-labelled cells. Ordinate: % of epithelial cells labelled only with ^{3}H. Abscissa: time interval (h) between [^{3}H]- and [^{14}C]thymidine injections.

G_2 period

The method of labelled mitoses (Quastler & Sherman, 1959) was used to determine the duration of the G_2 period. Hydra were pulse-labelled once with [^{3}H]thymidine and fed daily thereafter. At various times after the pulse, samples were taken to determine the percentage of mitotic epithelial cells which were labelled.

Fig. 6A gives the percentage of mitotic cells labelled for a period of 5 days after a single pulse labelling. There are 2 unusual features to the results: (1) the minimum length of G_2 is about 24 h, which is very long compared to the duration of G_2 generally found in other cell types (Cleaver, 1967); and (2) the length of G_2 is variable since the percentage of mitotic figures which were labelled never exceeded 30%. If the duration of G_2 were constant for all epithelial cells, the percentage labelled mitotic figures should reach 100% at G_2 hours after pulse labelling.

Because the histogram of labelled mitoses has no obvious maximum or minimum, it is not possible directly to determine at what time all the initially labelled epithelial cells have undergone mitosis or, in other words, what the maximum length of G_2 is. However, by integrating the area under the histogram of labelled mitoses, the time can be estimated at which the cumulative number of labelled mitoses equals the number of epithelial cells initially labelled. Before performing the integration, each value in Fig. 6A must be corrected for the mitotic index at that time, the exponential growth of the cell population which occurred subsequent to the pulse labelling, and the duration of the labelled mitosis sample (8 h) relative to the duration of mitosis (1·5 h).

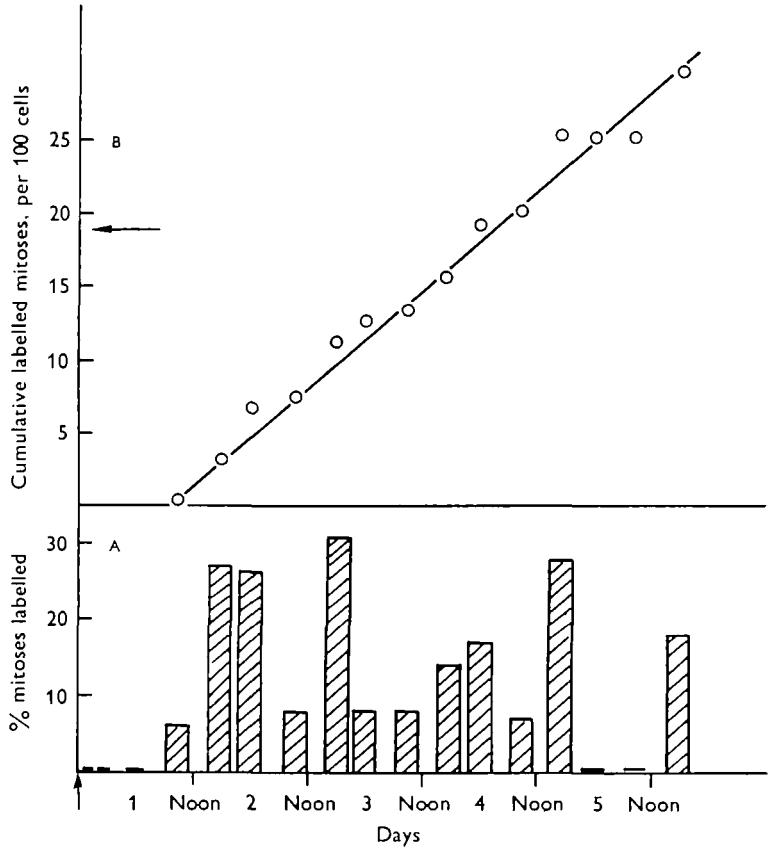

Fig. 6. Determination of G_2 duration by the technique of labelled mitoses. 50 hydra were pulse-labelled with [^{3}H]thymidine (50 μCi/ml) at start of experiment (arrow, on abscissa) and fed daily thereafter as usual. Macerations were prepared from 3 hydra at each time shown, stained with Feulgen, and epithelial cell mitotic figures mapped on the slides prior to autoradiography. A, % of mitotic figures labelled with [^{3}H]thymidine as a function of time after pulse-labelling. B, cumulative sum of the number of labelled mitoses expressed per 100 cells of the initial population; each point on the curve was calculated from the corresponding value in A as described in text. The arrow on the ordinate indicates the labelling index at the start of the experiment.

Fig. 6B shows the accumulation of labelled mitotic figures with increasing time after pulse labelling. When the cumulative number attains the value of initially labelled cells – namely 19% – at 3·5 days, then the cells labelled in the pulse have divided once on the average. The linearity of the cumulative labelled mitoses curve (Fig. 6B) beyond the value of 19% indicates that the second generation of labelled cells has started to enter mitosis before the first generation has finished. This indicates that the epithelial cell G_2 period varies from 24 to at least 72 h.

Mitosis

The duration of mitosis (t_m) can be calculated from the relationship

$$t_m = \frac{MI \times T}{\ln 2}$$

where MI is the mitotic index and T is the population doubling time (Cleaver, 1967). The daily average mitotic index for epithelial cells is 1·5% (derived from data in Fig. 4) and the population doubling time is about 3 days (Figs. 2, 6). Hence the duration of mitosis is about 1·5 h. This includes stages from middle prophase through late telophase.

DISCUSSION

Total epithelial cell cycle

The total duration of the hydra epithelial cell cycle can be estimated in several ways: (1) The sum of the durations of the separate phases (G_1 = 0, S = 12–15, G_2 = 24 to at least 72, and M = 1·5 h) indicates a duration of 40 to at least 85 h. Estimating the average G_2 to be about 55 h yields an average cell cycle estimate of 72 h. (2) The continuous labelling experiment (Fig. 2) indicates that the maximum cell cycle time is about 85 h. (3) The cumulative labelled mitoses curve (Fig. 6B) indicates that most pulse-labelled cells have divided within 85 h after the pulse. Since G_1 = 0 h, this indicates a maximum cell cycle of about 85 h.

The results obtained above with all 3 methods are in good agreement. They indicate that the maximum cell cycle duration is about 3·5 days and that the average is somewhat shorter. The doubling time of these hydra's tissue is about 3·3 days (Fig. 1), slightly greater than the average epithelial cell doubling time. This small 'excess' production of epithelial cells presumably accounts for the 10–15% (per doubling time) loss of epithelial cells due to sloughing at the ends of tentacles and the basal disk (Campbell, 1967*b*). The fact that the epithelial cell population grows at a rate equal to the growth rate of hydra makes unnecessary any significant production of epithelial cells by differentiation as suggested by Brien & Reniers-Decoen (1949) and Lentz (1966).

Distributed growth versus growth zone

The concept of growth distributed among all parts of hydra tissue requires that all epithelial cells divide and that the division rate be approximately equal to the growth rate of hydra. The concept of a growth zone predicts a fraction of rapidly dividing epithelial cells and a large fraction of non-dividing cells. Our results confirm the requirements of the distributed growth hypothesis, namely that more than 90% of the epithelial cells proliferate at a rate equal to the tissue growth rate. The few non-dividing cells are found in the peduncle-basal disk region and the tentacles (Campbell, 1967*a*). Thus our data extend the accumulating evidence (see Webster, 1971) for distributed growth to another species of hydra.

The wide variability in epithelial cell cycle times suggests the possibility of several populations of epithelial cells. Were such populations of rapidly and less rapidly dividing epithelial cells localized in hydra they might have significant effects on morphology. Fig. 2 shows that no such populations can be detected by dividing the body column into 3 segments along its main axis. (The proliferating fraction of cells in the peduncle-basal disk is completely labelled at 3 days, similar to cells elsewhere in the body column.) Furthermore, a continuous-labelling experiment performed on developing buds indicates that also here epithelial cell proliferation proceeds at virtually the same rate as in the body column (C. N. David, unpublished).

Variable duration of G_2 period

The results in Fig. 6 indicate that epithelial cells have a long minimum G_2 (24 h) and that G_2 can vary from 24 to at least 72 h. This situation is unusual for animal cells. Normally such cells have a G_2 period of short and fixed duration (Cleaver, 1967) although Gelfant (1963) has found that small numbers of cells in many tissues may be arrested in the G_2 phase.

Hydra epithelial cells are not 'blocked' in G_2 since they are actively proliferating. However, the sharp rise in mitotic activity following feeding (Fig. 4, also Campbell, 1967*a*) together with the variable G_2 period suggests the possibility of some form of mitotic control. Epithelial cells may have a defined minimum G_2 period (probably 24 h) followed by an indefinite G_2 period from which cells randomly are selected to undergo mitosis in response to a feeding stimulus (perhaps the stretching of the epithelial tissue by food in the gastric cavity). In support of such a hypothesis we have found that in poorly fed or starving hydra the G_2 period can be at least 6 days long.

In hydra epithelial cells the G_2 period has substituted for G_1 as the principal interphase state. This means that DNA synthesis and cell division are not necessarily tightly coupled. One advantage this confers on the tissue is the possibility of a rapid response to morphogenetic influences by division. The occurrence of a long G_2 period is very important to recognize in experimentation, since blocking DNA synthesis might not block mitotic activity in epithelial cells for several days.

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